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PKD is recruited to sites of actin remodelling at the leading edge and negatively regulates cell migration

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Abstract Protein kinase D (PKD) has been implicated in the regulation of cell shape, adhesion, and migration. At the leading edge of migrating cells active PKD co-localizes with F-actin, Arp3 and cortactin. Platelet derived growth factor (PDGF) activates PKD and recruits the kinase to the leading edge, suggesting a role for PKD in actin remodelling. In support of this, PKD directly interacts with F-actin and phosphorylates cortactin in vitro. Interference with PKD function by overexpression of a dominant negative PKD or by PKD-specific siRNA enhanced cell migration, whereas cells overexpressing PKD wild type displayed reduced migratory potential. Taken together, these data reveal a negative regulatory function of PKD in cell migration.
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Keywords: Actin remodelling; Cell migration; F-actin interaction; Phosphorylation; Leading edge

1. Introduction

The Protein kinase D (PKD) family of serine/threonine kinases consists of three structurally related isoforms: PKD1, PKD2 and PKD3. They are broadly expressed in most tissues and have been implicated in various fundamental cellular functions (reviewed in [1,2]). PKDs display dynamic changes in intracellular localization, they are recruited to the trans-Golgi network (TGN) and the plasma membrane in a diacylglycerol (DAG) dependent manner, a prerequisite for transphosphorylation and activation by nPKCs [3,4], but may also shuttle between cytosol and nucleus [5,6].

Several studies indicated a role for PKD in regulation of cell shape, motility, and adhesion. PKD was proposed to form a complex with the actin-binding protein cortactin and the focal adhesion protein paxillin in breast cancer cell invadopodia [7]. However, the specific role of PKD in this complex remained undefined. Another link of PKD to cell migration is given with its substrate Kidins220 which is localized at neurite tips and growth cones of PC12 cells [8]. In motile immature dendritic cells, Kidins220 was localized to a raft compartment of membrane protrusions at the leading edge of migrating cells [9]. Co-localization of PKD in these structures supported a role for PKD in cytoskeletal reorganization and cell shape modulation. Locomoting cells exhibit a constant retrograde flow of membrane proteins from the leading edge of the lamellipodia backwards, which when coupled to substrate adhesion might drive forward movement of the cell. In NIH3T3 fibroblasts, a kinase dead, dominant negative mutant of PKD1 specifically inhibited retrograde flow of surface markers as well as filamentous actin (F-actin) [10]. The observed inhibition of cell motility was strictly coupled to the block of vesicle transport from the TGN to the plasma membrane [10], a central function of PKD [11]. In another study, PKD mediated phosphorylation of the cell adhesion molecule E-cadherin was associated with altered cellular aggregation and motility of prostate cancer cells [12]. Inhibition of PKD1 activity by Gö6976 resulted in decreased cellular aggregation, whereas overexpression of PKD1 increased cellular aggregation and decreased cellular motility [12]. Taken together, a role of PKD in cell motility becomes apparent, supposedly involving quite distinct, direct, and indirect mechanisms of action. In order to further elucidate PKD's role as a regulator of cell migration we here studied PKD localization and interacting molecules in murine pmi28 myoblast cells [13], murine B16-F1 melanoma cells and human Panc89 pancreas adenocarcinoma cells [14]. Our data provide evidence that in each of these different cell types, PKD is recruited to sites of actin remodelling at the leading edge of migrating cells. We further show that PKD directly interacts with F-actin, phosphorylates cortactin and negatively regulates cell migration.

2. Materials and methods

See Supplementary data.

3. Results and discussion

The mouse myoblast cell line pmi28 [13], expresses all three PKD isoforms as detected by Western blotting and immunofluorescence microscopy, revealing a similar broad cytoplasmic distribution of the PKD proteins with the expected perinuclear enrichment and a discrete staining of plasma membrane stretches (Fig. S1). To investigate the localization of active PKD we performed a staining with an antibody specific for autophosphorylated PKD1 and PKD2 (pS910), showing that the plasma membrane located PKD is in an active state (Fig. 1A). Co-staining for F-actin and Arp3 indicated co-localization of active PKD with both of these markers at sites of membrane protrusions, representing lamellipodia of migrating

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Abbreviations: DAG, diacylglycerol; GFP, green fluorescent protein; PKC, protein kinase C; PKD, protein kinase D; PDGF, platelet derived growth factor; TGN, trans Golgi network

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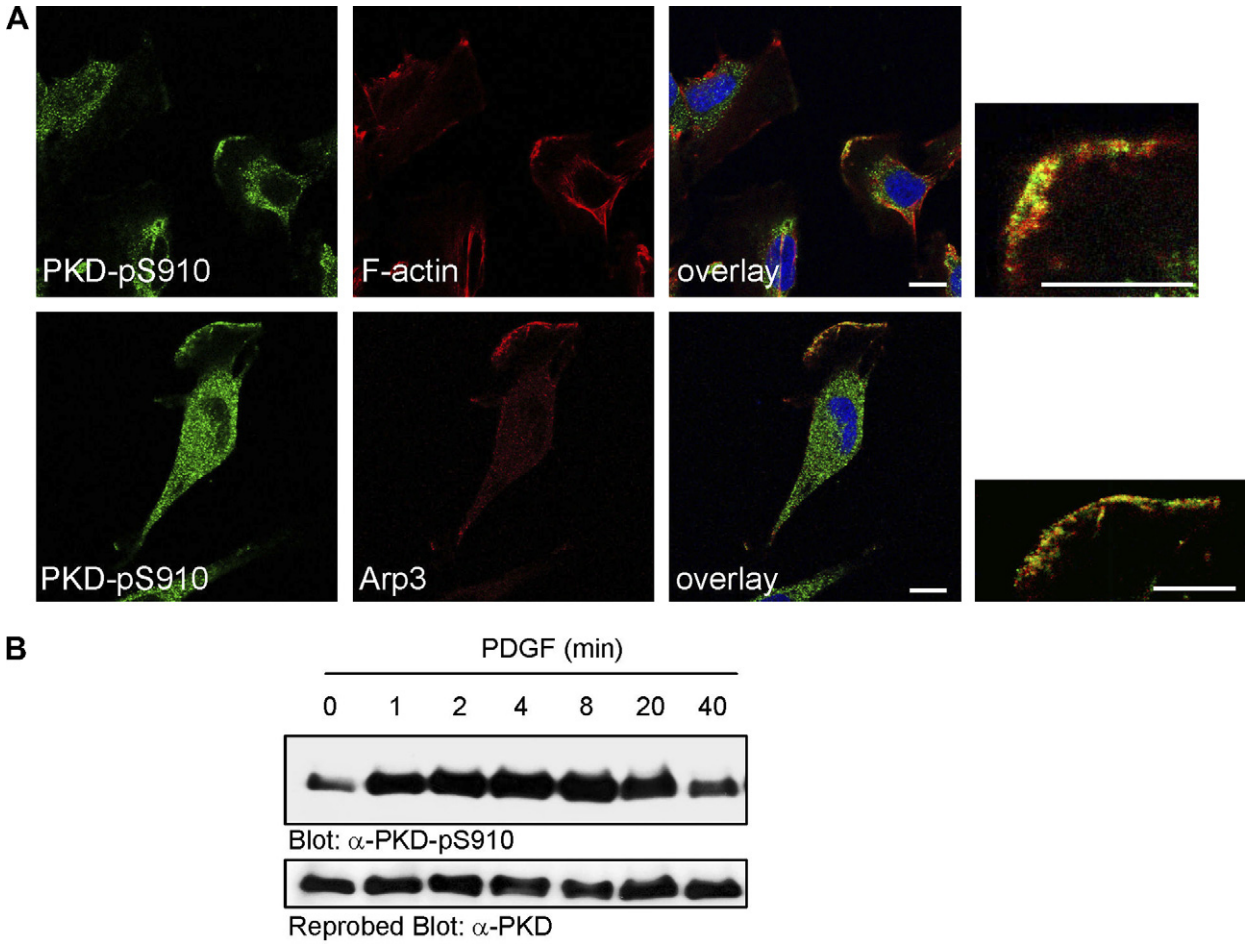

Fig. 1. PKD is recruited to sites of actin remodelling. (A) pmi28 cells stained with antibodies specific for phosphorylated PKD (pS910) (top and bottom) and Arp3 (bottom) or with rhodamin-phalloidine (top) to visualize F-actin. Nuclei were stained with Draq5 (blue). (B) pmi28 cells were treated with PDGF-BB for the indicated time points. PKD was monitored by Western blotting with a phospho-specific pS910 PKD antibody, and after stripping with a PKD-specific antibody. (C) pmi28 cells treated with PDGF-BB and stained for pS910-PKD and F-actin. Arrows indicates co-localization of PKD and F-actin at the leading edge. Scale bar 10 μm. (D) pmi28 cells were treated as described in (A). 100 cells per coverslip were quantified for co-localization of active PKD with F-actin at the leading edge. Results for positive double stained structures were calculated in percentage of total cells analyzed. The mean values were calculated for three independent experiments and are shown in a graphic with associated errors (S.D.). Statistically significant differences according to Students paired *t*-test are marked with an asterisk (*). All images shown are single confocal sections.

pmi28 cells (Fig. 1A). PKD responds to a variety of external signals, including growth factors such as Platelet-derived growth factor (PDGF) [15]. PDGF has clearly been shown to modulate the formation of lamellipodia [16]. In this line, we wanted to investigate whether PDGF-stimulation induces a recruitment of PKD to newly formed lamellipodia. First, we analyzed the kinetics of PDGF-mediated PKD activation using autophosphorylation as an indicator. PDGF-BB treatment induced rapid PKD activation peaking at 4–8 min and returning to basal levels 40 min after onset of PDGF-BB stimulation (Fig. 1B). Next, we monitored the localization of active PKD in PDGF-induced F-actin positive membrane protrusions over time by immunofluorescence microscopy (Fig. 1C) and quantified the amount of cells demonstrating co-localization of both proteins. The data summarized in Fig. 1C, lower panel, indicate a rapid recruitment of active PKD into PDGF-induced membrane protrusion sites. Taken together, these data suggest an active role of PKD at sites of actin remodelling.

For several protein kinase C (PKC) isoforms, in particular PKCδ, PKCε, PKCβII and PKCζ [17–20] direct F-actin binding in vitro has been demonstrated. Although the PKD sequence contains no F-actin binding motifs similar to those present in PKCs [21], the co-localization data (Figs. 1 and 2) prompted analysis on potential PKD binding to F-actin. To analyze direct interaction of PKD with F-actin, co-sedimentation assays using ectopically expressed PKD1-variants or control lysates from HEK293 cells were performed. As shown in Fig. 2A, both, PKD1-GFP WT as well as PKD1-KD-GFP bound to F-actin in vitro. The known F-actin-binding protein cortactin [22,23] was used as positive control, while green fluorescent protein (GFP) served as respective negative control for the assays. Of note, also PKD2 demonstrated binding to F-actin in this assay (Fig. 2A). In order to discriminate between an indirect PKD recruitment via interaction within multiprotein complexes at the actin cytoskeleton, or by direct binding to F-actin, we incubated purified PKD1 proteins with F-actin. Both, PKD1 and PKD1-KD, were detected in the pellet fraction (Fig. 2B), indicating that PKD1 directly binds to F-actin in vitro, independent of its kinase activity. To map the region responsible for mediating PKD binding to F-actin, several

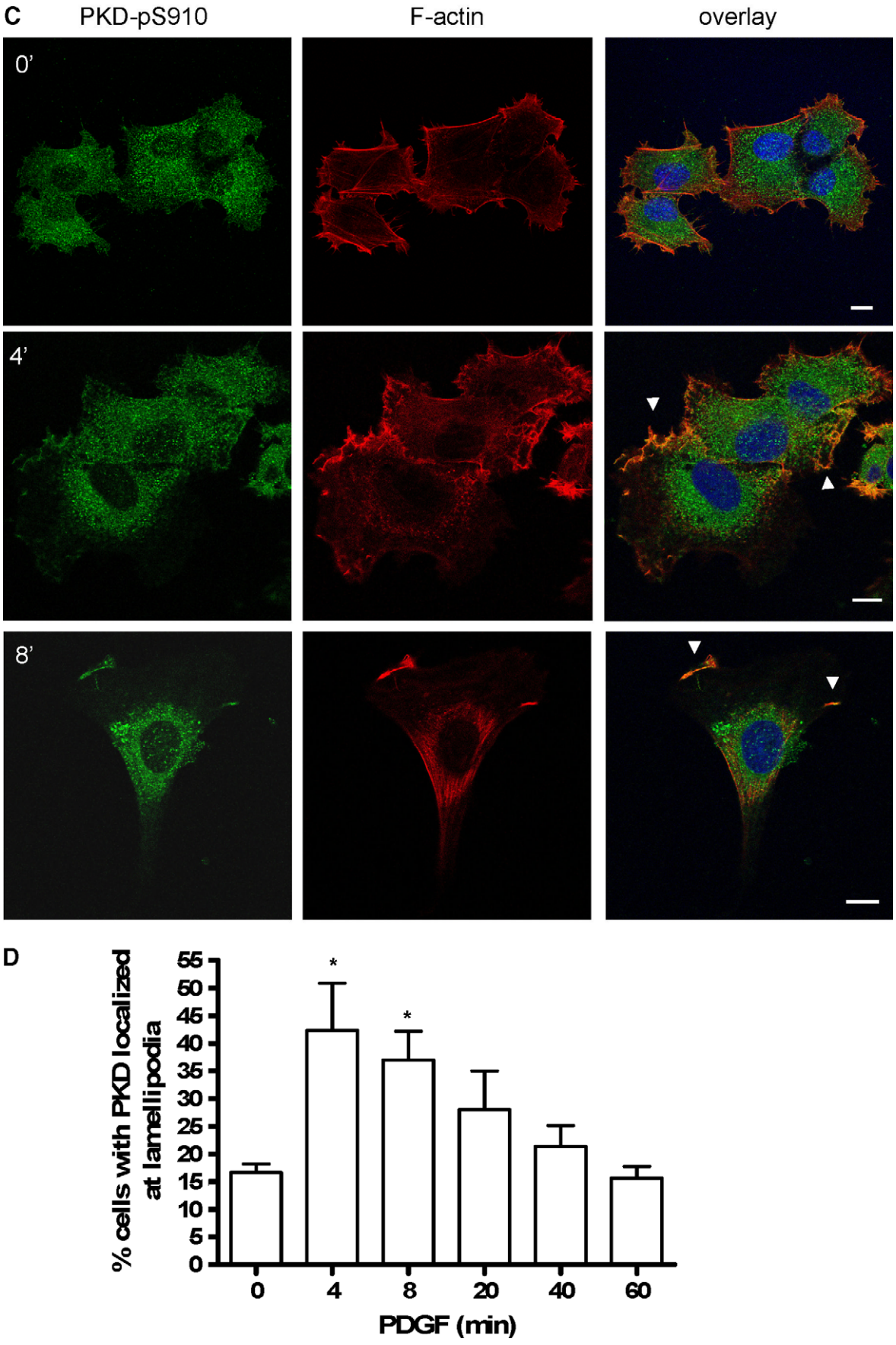

Fig. 1 (*continued*)

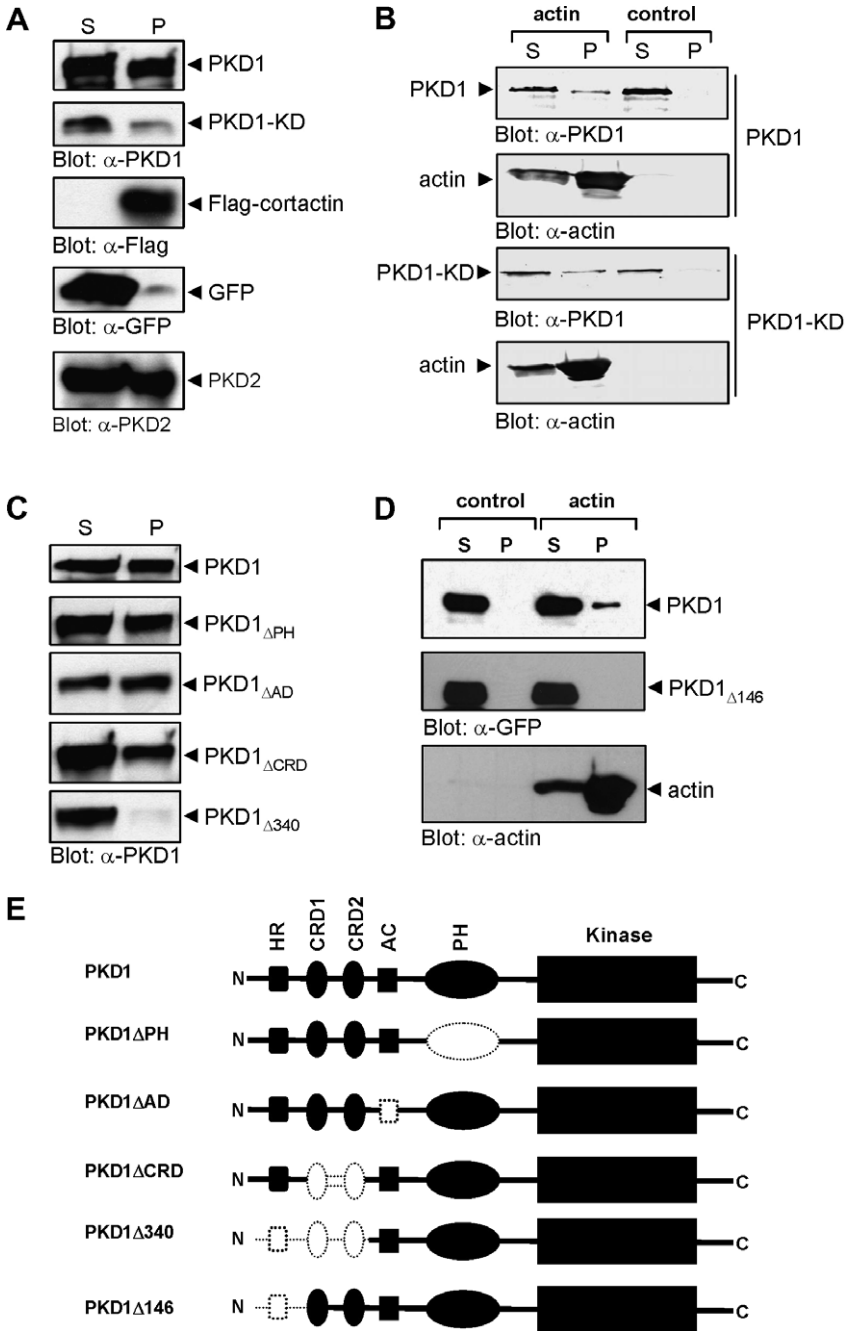

Fig. 2. PKD directly interacts with F-actin in vitro. (A) F-actin co-sedimentation assay with transiently expressed proteins. PKD proteins were detected by Western blotting with PKD-specific antibodies. GFP-Vector and FLAG-Cortactin were included as negative and positive controls, respectively. (B) F-actin co-sedimentation assays with purified PKD1 and PKD1-KD proteins. Assays without F-actin were included as respective controls. Actin was detected by Western blotting with an actin-specific antibody. (C, D) F-actin co-sedimentation assay to map the F-actin binding domain of PKD1 using total cell lysates from cells expressing PKD1-GFP and respective PKD1-GFP deletion mutants. Assays without F-actin were included as respective controls. PKD proteins and actin were detected by Western blotting with GFP- and actin-specific antibodies, respectively. S, supernatant; P, pellet fraction. (E) Schematic representation of PKD1 WT and deletion mutants. HR, hydrophobic region; AC, acidic domain; PH, pleckstrin homology domain; CRD, cysteine rich domain; kinase, kinase domain.

PKD deletion mutants as indicated in Fig. 2E were subjected to F-actin co-sedimentation assays. A PKD1 mutant with a deletion of amino acids 1-340 (PKD1Δ340-GFP) completely failed to co-sediment with the F-actin fraction, indicating that the N-terminal regulatory region of PKD is involved in F-actin binding. Deletion of the PH domain (ΔPH), the acidic domain (ΔAD) or the DAG-binding cystein rich domain (ΔCRD) did not affect F-actin binding in vitro (Fig. 2C). Accordingly, the F-actin-binding motif of PKD1 resides within the N-terminus comprising amino acids 1–146. To test this, we created a GFP-tagged PKD1 protein lacking the first 146 amino acids (PKD1Δ146-GFP, Fig. 2E) and tested whether this protein was capable of binding to F-actin. PKD1Δ146 failed to co-sediment with the F-actin fraction, thus confirming our conclusions (Fig. 2D). Based on the fact, that PKD2 is also capable of binding to F-actin, we suggest

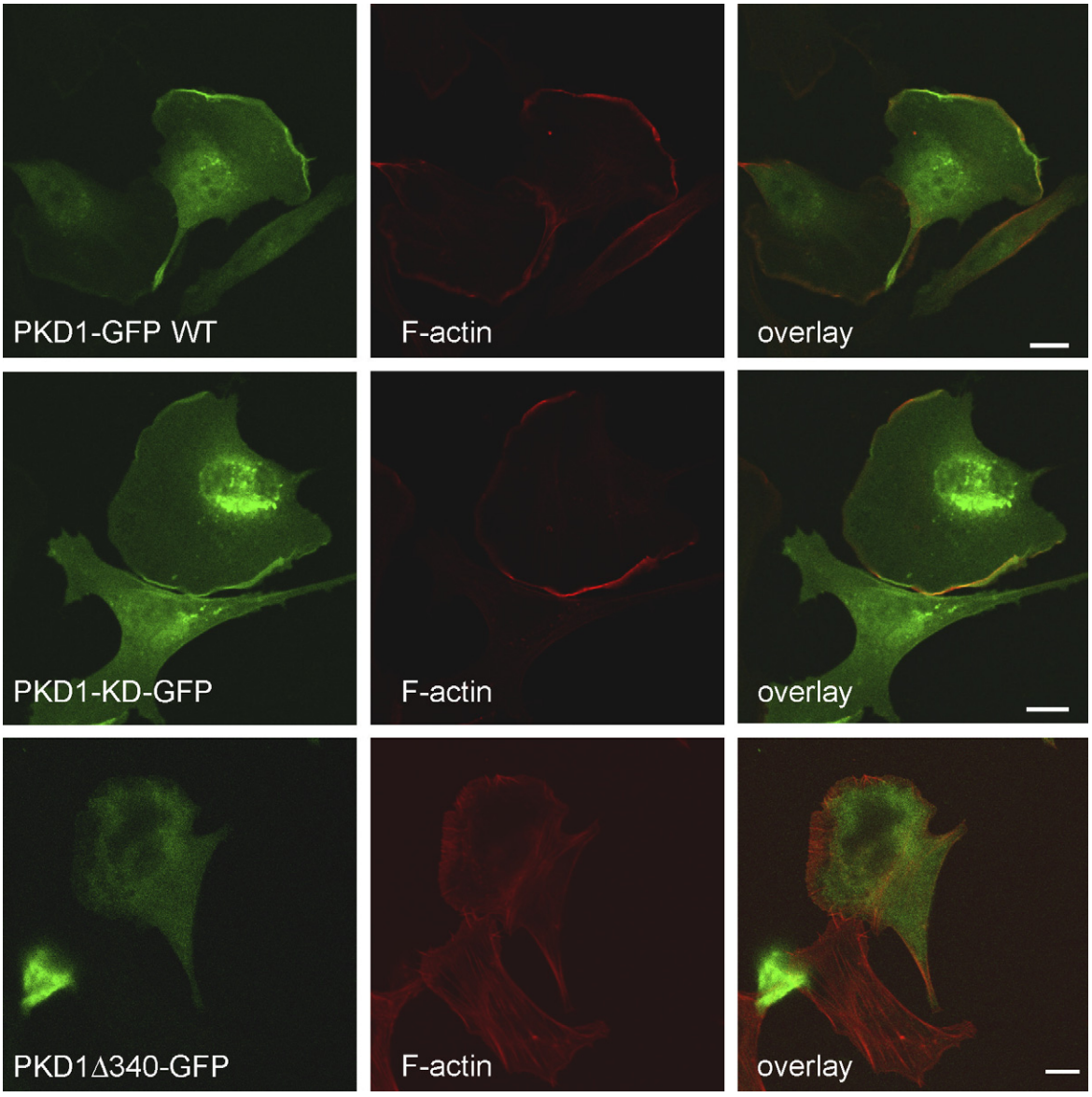

Fig. 3. PKD recruitment is independent of its kinase activity. B16-F1 cells transiently expressing PKD1-GFP, PKD1-KD-GFP and PKD1Δ340-GFP proteins were seeded at 30% confluency on laminin-coated coverslips. Cells were treated with aluminofluoride for 20 min, fixed and stained with rhodamin-phalloidine to visualize F-actin. Shown are single confocal sections. Scale bar 10 μm.

that the F-actin binding motif is conserved throughout PKD family members.

To corroborate these findings, we studied mouse B16-F1 melanoma cells which are used as an in vitro model for aluminofluoride (AlF) induced lamellipodia formation [24]. We studied co-localization of PKD1-GFP wild type (WT), a kinase dead (KD), dominant negative mutant, PKD1-KD-GFP and PKD1Δ340-GFP with F-actin, respectively at the lamellipodium after AIF treatment. Both, PKD1-WT and PKD1-KD were equally recruited into the leading edge of B16-F1 cells (Fig. 3). However, PKD1Δ340 was not found in areas of actin remodelling at the plasma membrane. These findings show that PKD recruitment to the leading edge of migrating cells was dependent on the aminoterminal domain and independent of its catalytic activity. This is in accordance with the present model of stepwise PKD activation, requiring a DAG dependent membrane recruitment of catalytically inactive PKD and a subsequent membrane bound nPKC mediated transphosphorylation [3,4].

However, it is possible that in addition to a direct F-actin binding, PKD1 also indirectly associates with F-actin via known actin binding proteins. In this line, it should be noted, that PKD1 has been reported to directly associate with cortactin [7] which is enriched in lamellipodia and implicated in the nucleation and/or stabilization of cortical branched F-actin networks [22]. In Panc89 cells which showed strong expression of PKD [25] endogenous PKD displayed cytosolic distribution with perinuclear enrichment and was also detectable at discrete plasma membrane protrusions, where it co-localized with F-actin (Fig. 4A, upper panel) and cortactin (Fig. 4A, lower panel). An interaction between PKD and cortactin at the leading edge might involve phosphorylation of cortactin. We therefore expressed Flag-cortactin together with GFP, PKD1-GFP WT, and PKD1-KD-GFP and subjected the proteins to an in vitro

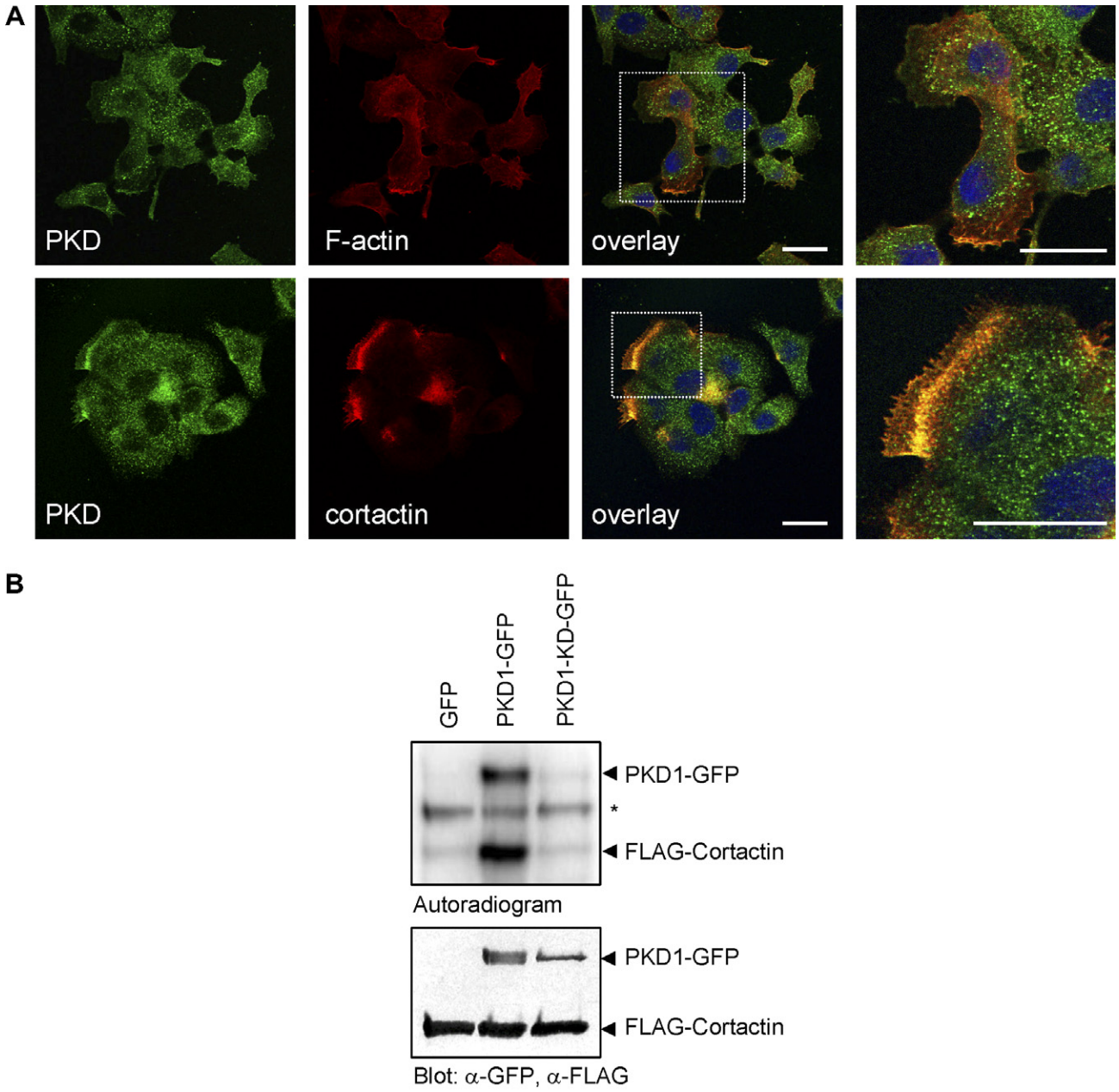

Fig. 4. PKD directly phosphorylates cortactin in vitro. (A) Panc89 cells were stained with an antibody specific for PKD together with rhodamin-phalloidine to visualize F-actin (upper panel) and an antibody specific for cortactin (lower panel), respectively. Shown are single confocal sections. Scale bar 20 μm. (B) Flag-cortactin and the respective GFP-proteins were co-precipitated from cell lysates with anti-Flag and anti-GFP antibodies and subjected to kinase reaction. Incorporation of radioactive phosphate was analyzed using a PhosphoImager (top), followed by immunoblotting with Flag-and GFP-specific antibody to verify equal loading. The bands marked with asterisks are non-specific.

kinase assay. We detected phosporylated Flag-cortactin in the presence of PKD1-GFP WT, but not in the presence of PKD1-KD-GFP or the GFP vector control (Fig. 4B), indicating a specific phosphorylation of cortactin by PKD.

In order to assess functional consequences on cell motility of PKD binding selectively at sites of dynamic actin remodelling, stable Panc89 cell lines expressing PKD1-GFP wild type and PKD1-KD-GFP were isolated by G418 selection and multiple rounds of cell sorting for GFP-expressing cells. Immunofluorescence flow cytometry (data not shown) and Western blot analyses (Fig. 5A, right panel) of the stable cell lines verified transgene expression. PKD1-WT and PKD-KD expressing cells show a similar doubling time and a slightly enhanced proliferation capacity compared to the GFP control (data not shown). Migration assays were performed using Transwell filter inserts. Parental (not shown) and GFP vector control cells showed no difference in migration behaviour. However, overexpression of both PKD1 WT and PKD1-KD resulted in highly significant ($P < 0.0001$) changes, suppressing and enhancing, respectively, the migration of Panc89 cells through transwell filters (Fig. 5A, left panels). In a total of 9 experiments performed on average PKD1 WT reduced the number of migrating cells to 25.5% and PKD1-KD enhanced the number of migrating cells to 234.4% of GFP vector control cells. Of

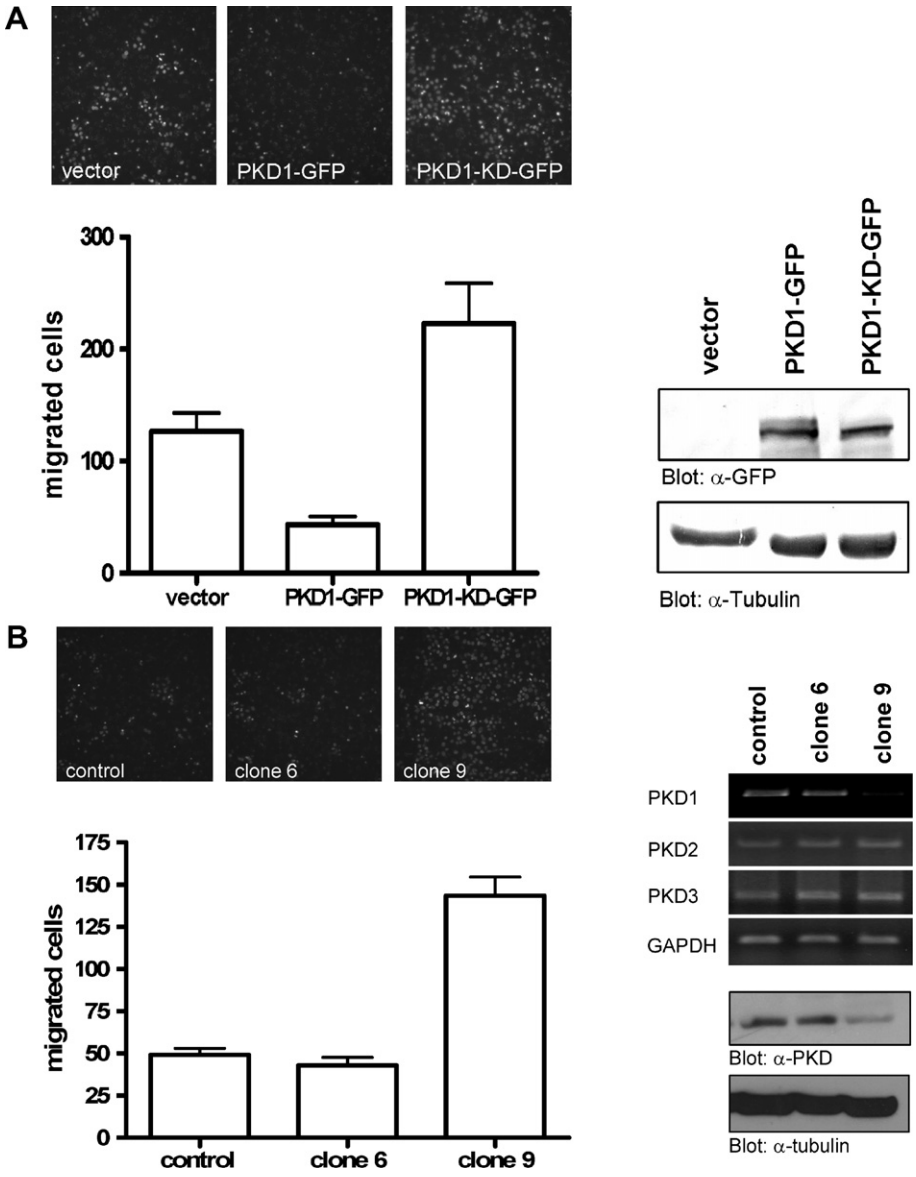

Fig. 5. PKD negatively regulates cell migration. (A, B) Panc89 cells stably expressing GFP, PKD1-GFP WT and PKD1-KD-GFP (A) or cells stably transfected with pSuppressor-PKD1 plasmid (B, clones 6 and 9) or parental Panc89 cells (A, B, control) were seeded on Transwell filters and migration was induced by a FCS gradient of 1–10% FCS. DAPI stained nuclei of migrated cells were used for quantification (upper panel). Results were calculated as median number of migrated cells/visual field (lower panels). The assay is representative of at least 3 experiments performed under the same settings. Expression of transgene was controlled by Western blotting of total cell lysates with a GFP-specific antibody (A, right panel). Semi-quantitative RT-PCR of PKD isoforms 1/2/3 and GAPDH from parental Panc89 cells and pSuppressor-PKD1 clones 6 and 9 (B, right panel). Western blotting of total cell lysates from control and clones 6 and 9 cells with a PKD1 specific antibody (B, right panel). Detection of tubulin verifies equal protein loadings (B, right panel).

note, these stable cell lines exhibited the same migration phenotype without a FCS gradient (data not shown).

To further scrutinize the functional role of PKD as a negative regulator of migration of Panc89 cells, we knocked down PKD1 expression by RNA interference. Because of the low efficiency of transient RNAi approaches in Panc89 cells we generated stable knock down clones using a G418 selectable pSuppressor construct coding for a short interfering RNA (siRNA) specific for the PKD1 isoform. During the selection process, the bulk of primary clones either stopped growing during the expansion, or detached from the surface, dying subsequently. Only 2 clones survived the selection process and the expansion to individual, stably growing cell lines. One of these (clone 9) presented as a PKD1-knockdown in semi-quantitative RT-PCR analysis and Western blot analysis (Fig. 5B, right panels), whereas clone 6 did not display reduced expression of PKD1, thus serving as a control. PKD2 and PKD3 specific RT-PCR verified a selective knockdown of the PKD1 transcripts in clone 9 PKD1-pSuppressor cells. Transwell migration assays (Fig. 5B, left panels) revealed strongly increased migration of the PKD1-knockdown clone ($P < 0.0001$), when compared to parental Panc89 cells, or clone 6, which retained normal PKD1 expression. These results are in accordance with the phenotype observed upon interference with PKD function by expression of a dominant negative PKD (Fig. 5A). Together, these data identify PKD as a negative regulator of cell

migration of Panc89 cells. Because a selective interference with PKD1 expression already accomplished a phenotype comparable to that observed with functional inhibition of potentially all PKD isoforms by dominant negative action, an isotype specific PKD function in this cell line appears possible.

Depending on the cell model studied, different mechanisms have been proposed through which PKD may influence cell motility [7,10,12]. It appears therefore of relevance that in the murine and human cells of distinct tissue origin studied here, in each case, active PKD was detectable in places directly involved in mediating cell motility, pointing to a common mechanism in these different cell types. The co-localization of PKD with components of the actin branching machinery, such as Arp3 (this study) and cortactin ([7] and this study) in living cells, supports a direct role of PKD in dynamic changes of the branched actin network. Our data also provide a first clue as to the underlying mechanism of PKD mediated regulation of cell migration through demonstrating direct F-actin binding of PKD in vitro, and selective co-localization with F-actin at the leading edge of migrating cells. Interestingly, PKD is not localized at actin stress fibers, implicating a compartimentalized interaction of PKD and F-actin. It is therefore conceivable, that interaction of PKD with the plasma membrane is a precondition for the direct interaction of both proteins bringing PKD close to F-actin at lamellipodia. Another explanation would be a recruitment of PKD by other factors to distinctive sites at the F-Actin network, where it might exert possible functions in the regulation of actin remodelling. In this context cortactin would be an ideal candidate to act as a scaffolding protein which could facilitate the recruitment of PKD downstream of a PDGF signal since both proteins interact [7]. Moreover, we here demonstrate that cortactin is a potential novel substrate for PKD at these sites. The role of cortactin as molecular scaffold in actin remodelling is regulated by tyrosine and serine/threonine kinases [26]. Phosphorylation of cortactin regulates its ability to activate N-WASP as well as F-actin binding capacity [27,28]. Recently, 17 novel phosphorylation sites have been identified in a mass spectrometry approach, the function of which is still unclear [29]. Based on our data, we hypothesize that PKD regulates the dynamics of actin turnover through phosphorylation of proteins of the actin polymerization machinery, such as cortactin. This may lead to a stabilization of the cortical actin network, thereby reducing migration potential.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.07.079. This section also cites Refs. [30,31].

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